

## Research Article

# Algal-Fungal Mutualism: Cell Recognition and Maintenance of the Symbiotic Status of Lichens

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**OPEN ACCESS****Abstract**

Lichens are specific symbiotic associations between photosynthetic algae or cyanobacteria and heterotrophic fungi forming a double entity in which both components coexist. Specificity required for the lichen establishment can be defined in this context as the preferential, but not exclusive, association of a biont with another, since the algal factor susceptible to be recognized is an inducible protein. Recognition of compatible algal cells is performed by specific lectins produced and secreted by the potential mycobiont. Some lectins from phycolichens and cyanolichens are glycosylated arginases which bind to an algal cell wall receptor, identified as a  $\alpha$ -1, 4-polygalactosylated urease. However, other ligands exist which bind other lectins specific for mannose or glucose. This implies that, after recognition of a potential, compatible partner, other fungal lectins could determine the final success of the association. Since the fungus can parasitize non-recognized partners during the development of the association, the success after the first contact needs of a set of algal cells, the number of which was sufficient to prevent that the death of a certain number of them makes fail the symbiosis. Fungal lectins act as chemo-tactic factors in such a way that algae and cyanobacteria move towards the hyphae, to acquire that critical size of the colony, by means of successive contractions and relaxation of the actomyosin cytoskeleton in absence of any motile appendages.

**Keywords**

- Actin
- Alga
- Chemotactism
- Cytoskeleton
- Fungus
- Lectin
- Lichens
- Recognition
- Specificity

**INTRODUCTION**

Since Schwendener [1] described lichens as a set of two organisms in symbiosis, it has been accepted this association as mutualistic in which the fungal partner uses the photo-assimilates from the photosynthetic biont, a green alga or a cyanobacteria, whereas the photo-ergonic partner benefits from the dampness that the fungus can retain in its mycelium. Probably, this is a too simplistic vision about the functionality and survival of this association, but it has come being accepted without too many objections during more than one century.

Nevertheless, certain observations and some experimental results seem to indicate that the lichen-forming fungus could conserve its "parasitic vocation" during the initial phases of the establishment of the association. This idea arose from some assays on lichen re-synthesis [2,3] in that the fungus, once initiated the contact with its compatible photobiont, might be submitted to a deprivation of nutrients to prevent the parasitic attack to the alga by the emission of haustoria followed by necrosis and death of the colony of algal cells. The fungus might "to feel" that those algae were the only disposable source of food and that, therefore, it might to attenuate its parasitic nature to preserve that potential reservoir of nutrients.

The term compatible photobiont needs an explanation. Since the first description of lichen as an alga-fungus association, it was known that every species of ascomycetes or basidiomycetes formed their symbiotic associations with a determined species of alga or cyanobacterium and not with any other, although the incompatible species would be phylogenetically nearby to the specific one.

Specificity can be defined in this context as the preferential, but not exclusive, association of a biont with another [4]. For example, the mycobiont of *Cladonia cristatella* produces squamules with different species of *Trebouxia*, displaying a selective behavior [3]. However, the mycobiont of *C. cristatella* cannot form squamules with green algae other than *Trebouxia*, showing high specificity [2]. Moreover, culture experiments performed to investigate the selectivity of the mycobiont of *Fulgensia bracteata* towards a variety of potential photobionts provide evidence for mycobiont selectivity and varying compatibility of the respective symbionts, which can be interpreted as a cascade of interdependent processes of specific and non-specific reactions between the symbionts [5]. In fact, Ahmadian [2] demonstrated that these "incompatible" algae were parasitically invaded by the fungus and suppressed from the media in which the re-synthesis was

trying to be carried out. Later, the concept of compatibility or incompatibility was related to the production of lectins by the fungal partner [6], phytohaemagglutinins that had been previously described, though not related to the recognition between lichen symbionts [7]. Lectins constitute a heterogeneous group of glycol proteins of non-immune origin with non - catalytic binding sites which are capable of recognizing and reversibly binding to specific saccharide moieties [8]. Since the nineteenth century, these proteins have been described mainly in dicot [9], but also in monocot [10], animals [11], bacteria [12], algae [13], yeasts [14], mushrooms [15], and several symbiotic associations such as *Rhizobium* - legume, mycorrhizae [16] and lichens [6,17].

This recognition system, based on the production of a lectin, a signal molecule produced by the fungal partner able to establish an affinity bond with a glycoprotein in the surface of the algal cell wall, depends on two determining facts that can be summarized in a following way:

1. A fungus able to form lichen can recognize a free - living alga with which it could contact in nature.
2. A lichenized fungus must recognize a newborn alga inside the lichen thallus, since the lichenized algae seasonally divide inside the thallus.

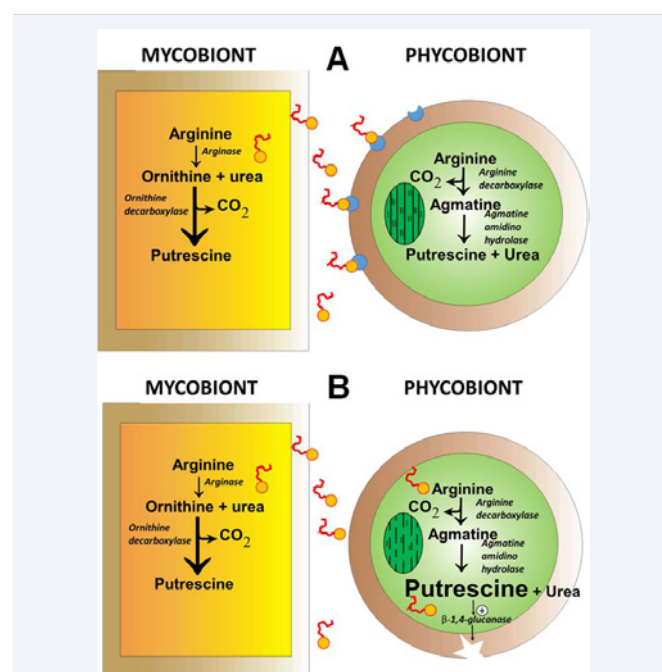
When algal cells multiply inside a growing thallus, daughter cells are enveloped by fungal hyphae, which recognize new cells as compatible [2]. Thus, recognition mechanisms are absolutely required, not only for de novo formation of new associations, but also for the maintenance of the symbiotic equilibrium in the lichen symbiosis. Ultra structural or re synthesis studies aiming to investigate the relationship between lichen symbionts in the lichen thallus suggested that such relationship might involve cell surface recognition factors [18].

### Fungal recognition of the compatible algae

Several phytohaemagglutinins have been isolated from a number of lichen species [7,19,20]. Lockhart et al., [21] found that these haemagglutinins bind to the appropriate photobiont, *Nostoc* isolated from *Peltigera canina* and *P. polydactyla*, two cyanolichen species. Moreover, a protein fraction isolated from the thallus of *Xanthoria parietina*, a chlorolichen species, labeled with fluorescamine showed strong binding ability to cultured phycobionts obtained from *X. parietina*, *Caloplaca auriantia* and *C. citrine* [22], whereas it did not bind to freshly isolated phycobionts from the same species or isolated and cultured algae from *Cladonia convoluta*, *Ramalina duriae* and *R. pollinaria*, species from taxonomic families different from Teloschistaceae. Protein binding occurred at the cell wall surface of algal cells, since labeled protein binds to algal cell wall ghosts. This ABP (Algal Binding Protein) is a polypeptide of 12.5 kDa [6] found in *Xanthoria* mycobionts cultured *in vitro* [23]. ABP is restricted to the hyphal cell walls along which it is distributed. A protein with similar binding properties was later obtained from the cyanolichen *Nephroma laevigatum* [24] and identified as a dimeric polypeptide composed by two monomers of 52 kDa and 55 kDa. Other lectins have been found in *Peltigera membranacea* [25] and in cephalodia of *P. apthosa* [26].

We have previously characterized an ABP partially purified

from *X. parietina* thallus as a glycosylated arginase, which hydrolyzes arginine into ornithine and urea [27]. Fluorescein - labeled ABP (glycosylated arginase) bound to the cell wall of isolated phycobionts of *X. parietina*, and its binding efficiency was strongly enhanced after culture of algal cells with 40 mM urea for 2 h. This treatment induced expression of a glycosylated urease located at the algal cell wall, which was almost identical to that secreted from thalli to media. This enzyme consists of a single polypeptide glycoprotein with a large polygalactose moiety. Binding of glycosylated arginase to urease inhibited enzymatic activities of both proteins. When glycosylated urease is lacking from the algal cell wall, fungal arginase is internalized, increasing the levels of algal putrescine, which promotes chloroplast disorganization, activation of glucanases and breakdown of the cell wall with loss of the protoplast [28,29]. This process has been then defined as algal incompatibility (Figure 1). This secreted arginase is unequivocally defined as a fungal enzyme



**Figure 1** Schematic representation of fungal recognition (A) of a compatible algal partner or fungal discrimination (B) by fungal lectins. The enzymes of arginine catabolism are compartmentalized in both symbionts. Whereas the fungus hydrolyzes arginine by a glycosylated arginase to produce ornithine, which is then decarboxylated to putrescine, the algal partner decarboxylates arginine to agmatine which is then hydrolyzed to putrescine and urea. The rate of putrescine production by the algal partner represents only a 20% that that achieved by arginase. A compatible alga stops the entry of fungal arginase by retaining the secreted enzyme on a superficial ligand, a glycosylated urease in the cell wall, by affinity binding and this impedes the increase of the level of putrescine inside the algal cells (A). On the contrary, the absence of the lectin ligand in the algal cell wall facilitates the entry of fungal arginase inside the algal cell. As a consequence, the amount of algal putrescine increases, activating a β-1, 4-glucanase that hydrolyzes the cell wall and produces the loss of the protoplast. In parallel, the increase of putrescine concentration disorganized thylakoids as well as the lamellar structure of the chloroplast and photosynthesis rapidly declines (B).

since activity assays in isolated partners show that arginase is preferentially produced by the fungal component and, in addition, isolated phycobionts in axenic culture do not secrete detectable arginase [30]. Moreover, phycobionts isolated from *X. parietina thalli* and loaded with 28 mM arginine in liquid media do not secrete arginase and their content in putrescine does not increase significantly, indicating that diamine synthesis via arginine decarboxylase and agmatine amidino hydrolase is regulated by a feedback mechanism [28].

Purified arginases secreted from *Evernia prunastri* and *X. parietina thalli* hydrolyze arginine in a  $Mn^{2+}$  - dependent reaction.  $Ca^{2+}$  cannot replace  $Mn^{2+}$ , but its addition to reaction mixtures in the presence of  $Mn^{2+}$  significantly inhibits arginase activity. Arginases from both lichen species also show lectin function, binding to the cell wall of both homologous and heterologous algae. Such binding is enhanced by both  $Ca^{2+}$  and  $Mn^{2+}$  and results in cyto agglutination, which is counteracted by  $\alpha$ -D-galactose [31]. The enzyme bound to its ligand shows to be inactive to hydrolyze of arginine [32]. The glycosidic moiety of the ligand for these lectins is uniquely composed of  $\alpha$ -D-galactose. Binding of lectins inhibits urease activity of the ligand, which is recovered after desorption of the lectin with  $\alpha$ -D-galactose. Data demonstrate ligand - dependent retention of the fungal lectin on the algal cell surface and this is consistent with a model of recognition of compatible algae (Figure 1), through which algal cells would form lichen with a lectin-secreting fungus only when those cells contain the specific ligand for the lectin in their cell walls. This is, lectin binding is used as a mechanism for ensuring specificity in the association [31]. Hydrolysis of the galactoside moiety of urease in intact algae with  $\alpha$ -1, 4-galactosidase releases high amount of D - galactose and impedes the binding of the lectin to the algal cell wall [32]. The production of glycosylated urease is restricted to the season of low temperature and short photoperiod in which algal cells divide and this as sure the recognition of new phycobiont produced after cell division by its fungal partner [33].

### Fungal recognition of compatible cyanobacteria

Rikkinen [34] described a model for signaling between symbionts in cyanolichens. Two types of signaling elicitors are known: general elicitors, which are usually substances associated with primary metabolism, and included glucans, chitin oligomers, glycopeptides, cell wall fragments and many phenolics, and specific elicitors that include proteins, peptides, syringolides and phenolic compounds. However, there is no experimental evidence for the involvement of phenolics in lichen symbiont recognition and, in addition; no specific phenolics are produced by cyanolichens. Cell recognition of trigger molecules involves Trans - membrane proteins, the inner membrane part of which may function as a kinase, transmitting signals to cellular mechanisms and resulting in interbiotic responses. Unfortunately, no experimental probes for this mechanism have yet been published.

Recognition of a compatible cyanobiont can also be affected by lectins. In ferns and bryophytes, the plant produces lectins that recognize sugar residues on the cell surface of a pre - symbiotic *Nostoc* [35]. Kardish et al., [24] conclude that lectins are involved in the control and regulatory processes of the symbiotic balance

in *Nephroma laevigatum* thallus. Lectins have also been isolated from the mycobionts of some *Peltigera* species [25,36]. In *P. aphthosa*, a lectin - recognizes compatible *Nostoc* cells at the initiation of cephalodium formation, and this process is highly specific [26]. The specificity of cyanobionts was confirmed in a study that attempted to introduce foreign *Nostoc* strains into cephalodia of *P. aphthosa* [37].

*Leptogium corniculatum*, a cyanolichen containing *Nostoc* as photobiont, produces and secretes arginase to culture medium containing arginine. This secreted arginase was pre - purified by affinity chromatography. Arginase was eluted from the beads with 50 mM  $\alpha$ -D-galactose. The eluted arginase binds preferentially to the cell surface of *Nostoc* isolated from this lichen thallus, although it is also able to bind, to some extent, to the cell surface of the chlorobiont isolated from *E. prunastri* [38]. Our experiments demonstrate that the model of chlorolichen can now be extended to cyanolichens.

Marohan et al., [39] have identified a lectin gene, lec-2, in the genome of *Peltigera membranacea*. The analysis of non synonymous versus synonymous rates for nucleotide substitutions indicates strong positive selection by amino acid replacements around the putative carbohydrate - binding pocket. This fact indicates changes in ligand binding. Positive selection of lec-2 seemed not be due to any variation in photobiont partners.

### Recognition of cyanobionts requires chemo tactic cell displacement

In non - haustorial lichens, the phycobiont cells, adhered to the surface of the mycobiont hyphae, are immersed in a gelatinous pod and they can slip to regulate in this way the intensity of light received [40,41]. In the context of the contact between a lichenized mycobiont and a free - living cyanobacterium, the possibility of the displacement to favor the first contact between potential symbionts seems to be possible. Hereby, the increase in the size of pool of cells in the surroundings of a hypha would compensate the death for some of them, accidentally recognized as incompatible cells.

To assure the success of the recognition process, lectin - recruited cells must move toward the hyphal surface to establish cell-to-cell adhesive contacts, which are mediated by the binding of lectins to free binding sites in the cyanobiont cell wall surface. One hypothesis is that the fungal lectin acts as a chemo attractant to recruit cyanobiont cells. Although the knowledge of cyanobacterial chemo taxis in plant or lichen associations is scarce, there is some evidence supporting this hypothesis. Dick and Stewart [42] described discrete fimbriae, non - flagellar appendages involved in cell motility, in *Nostoc* cyanobionts isolated from *P. canina* when grown on  $N_2$ . On the other hand, *Nostoc* cells from the liverwort *Blasia pusilla* appear as small, undifferentiated, motile (gliding) cells ("hormogonia") [43]. These cellular morphologies may promote cell dispersal. The reason for this morphological change remains unknown, although it can be hypothesized that the host produces a hormogonial-inducing-factor (HIF) that diffuses to the medium and interacts with *Nostoc* cells [44] modulating their morphology and/or motile properties. In this regard, Vivas et al., [38] suggest that *Nostoc* hormogonia produce the lectin receptor in cyanobiont isolated



from *L. corniculatum*. This is consistent with the putative role of hormogonia in the dispersal of *Nostoc* cells inside the thallus from the initial filament [34]. Several hormogonia separate from the pluricellular filament and migrate between fungal hyphae to establish in a new site inside the thallus. Due to the possibility of dispersion, hormogonia can hardly be visualized in recently isolated *Nostoc* from *P. canina thalli*, in agreement with our experimental results [45]. Mature, symbiotic cyanobacteria do not require continuous recognition by their fungal partner initially; however, recognition would be required to re-establish specific cell-to-cell contact if the initial contact with their mycobiont is lost. However, heterocysts,  $N_2$  - fixing cells, do not contain the fungal lectin receptor in their cell walls [45]. Consistent with this, a high frequency of heterocyst's follows increased hormogonia formation in free - living filaments in response to nitrogen - limited or starved *Nostoc* from *Anthoceros punctatus* [46]. In addition to diffusible HIF, the host plant produces other chemo attractants. Chemo attraction toward exudates or extracts of natural host and non - host plants and some sugars has been reported in *Nostoc* cells [47].

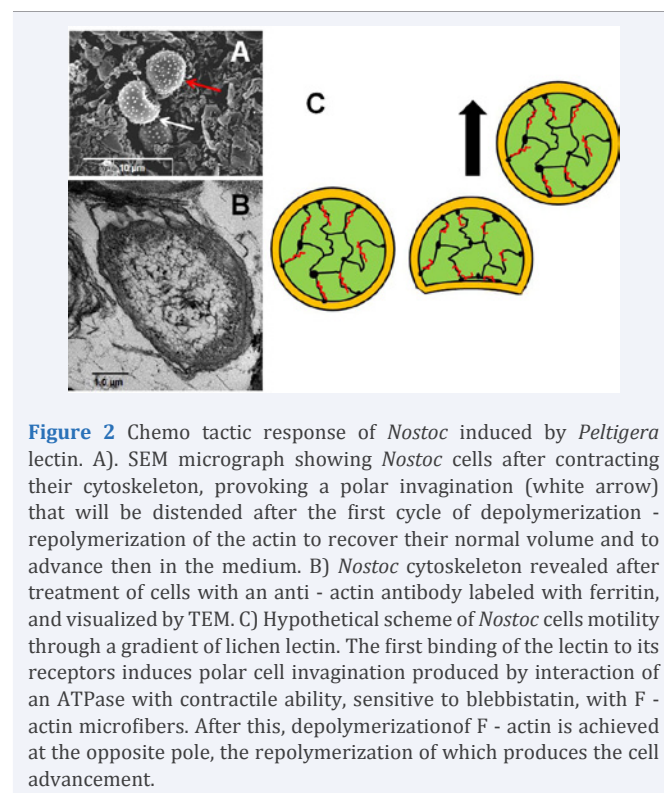
It is currently unknown whether formation of pili is required for cell gliding. Hormogonia from *Calothrix* express genes that promote pili differentiation during their formation [48]. Likewise, a type IV pilus system mediates pili formation on the surface of hormogonium of *N. punctiforme*; in this system, mutations in pili like genes altered surface piliation and reduced symbiotic competency [49]. However, cell motility without peripheral motile structures probably requires the assistance of cytoplasmic organelles.

Although cytoskeleton has not been well studied so far in prokaryotic cells, the presence of actin - like proteins has been reported in both bacteria and cyanobacteria developing functions related to chromosome segregation, cell motility in the absence of locomotive appendages and the maintenance the cell shape [50,51]. The shape of *Escherichia coli* [52, 53] and *Caulobacter* [55] is controlled by an actin - like protein, MreB, which also mediates segregation of *Caulobacter* chromosome [56]. Accordingly, the depletion of MreB causes defects in the cell shape of *Spirulina platensis* [57]. This protein contains conserved epitopes corresponding to actin regions known to interact with cross - linking proteins. They are also reported for *Anabaena* cylindrical and *A. variabilis* [58]. In a similar way to that found for bacteria, MreB depletion in *Anabaena* sp. drastically modifies the cell shape of this cyanobacterium [59].

A glycosylated arginase acting as a fungal lectin from *P. canina* is able to produce recruitment of cyanobiont *Nostoc* cells and their adhesion to the hyphal surface. This implies that the cyanobiont would develop organelles to motility toward the chemo attractant. However, *Nostoc* cells recently isolated from *P. canina* thallus do not reveal any motile, superficial organelles, although their surface was covered by small spindles and serrated layer related to gliding [60], when visualized by transmission electron microscopy. The use of S-(3,4-dichlorobenzyl) isothiurea, an inhibitor of MreB, blebbistatin, an inhibitor of myosin contraction, phalloidin and latrunculin A, inhibitors of depolymerization and actin - G polymerization, respectively, provide circumstantial evidence that actin microfilaments rather

than MreB, the actin - like protein from prokaryota, and probably, an ATPase which develops contractile function similar to that of myosin II, are involved in cell motility [61]. The absence of superficial elements (fimbriae, pili or flagellum) related to cell movement and the appearance of sunken cells during or before movement, verified by scanning electron microscopy (Figure 2), support the hypothesis that the motility of lichen cyanobionts could be achieved by contraction-relaxation episodes of the cytoskeleton induced by fungal lectin act as a chemo attractant [60]. Moreover, the inhibition of chemo taxis produced by the combined action of phalloidin and blebbistatin is largely reversed by GTP and some of its analogs, as well as by cyclic AMP. Movement implies a rearrangement of the cytoskeleton causing cell polarity, which is, in turn, inhibited by phalloidin and latrunculin A, as revealed by confocal microscopy. F-actin fibers composing *Nostoc* cytoskeleton have been visualized by immune cytochemical techniques associated with transmission electron microscopy [61].

Similar movements in unicellular eukaryotes require actin and myosin to generate contraction and relaxation along the chemotactic axis. We also provide evidences for prokaryotic actin - like and myosin-like proteins in the cyanobiont *Nostoc* sp. using cross-reacting antibodies against  $\alpha$ - and  $\beta$ -actin and non - muscle myosin II light and heavy chains, and two - dimensional gel electrophoresis to determine the iso electric point (IP) of the actin - like protein. Actin antibodies bind to a single reactive *Nostoc* polypeptide of an approximate molecular mass of 50 kDa, similar to eukaryotic actin. The myosin light chain antibody reacts with a *Nostoc* protein with an apparent molecular weight of 20 kDa and another of 48 kDa. Immuno precipitation of cell free extracts using anti - heavy chain myosin separately yields only one signal



corresponding to a protein of a molecular weight around 200 kDa. Our results are consistent with the possible existence of protein homologues of actins and myosins in cyanobionts of *P. canina*, suggesting the existence of an actin-like apparatus that supports chemo tactic swimming [62].

## CONCLUSION

The specificity between a fungus and an alga in the establishment of the lichen symbiosis is based on the production of fungal lectins that require specific ligands produced by the algae. The absence of these proteins, while their synthesis is repressed, determines the incompatibility of the alga to form the association. Fungal lectins also act as factors of chemo tactic attraction for the algae that move towards the hyphae and increase therefore the percentage of surviving algae after the first contact.

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